

The use of the [1,2-¹³C]acetate recovery factor in metabolic research

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Luc J. C. van Loon · Rene Koopman
Patrick Schrauwen · Jos Stegen
Anton J. M. Wagenmakers

The use of the [1,2-¹³C]acetate recovery factor in metabolic research

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Abstract To provide guidelines on the correct application of the acetate recovery factor in metabolic research, we investigated the influence of exercise intensity and infusion protocol on [1,2-¹³C]acetate label recovery during exercise. Eight cyclists were studied during [1,2-¹³C]acetate infusion for 1 h at rest followed by three 30-min stages of cycling exercise at a workload of 40, 55 and 75% maximal workload (W_{\max}), respectively (protocol 1). Four cyclists were subsequently studied following [1,2-¹³C]acetate infusion in three separate trials while cycling at the same workloads but in the absence of any pre-exercise infusion period (protocol 2). Finally, we observed the cyclists during [1,2-¹³C]acetate infusion at a 40% W_{\max} workload after 4 h of pre-exercise infusion (protocol 3). Acetate recovery increased from 13.7 (0.4)%, after 1 h of rest, to a plateau value of 75.1 (2), 91.2 (0.7) and 101 (2)% during exercise at 40, 55 and 75% W_{\max} workloads, respectively. In protocol 2, without prior infusion time, fractional label recovery was substantially lower at each separate workload. In contrast, when applying an extensive pre-exercise infusion period of 4 h, acetate recovery rates were substantially increased compared to the values observed in protocols 1 and 2 during exercise at a 40% W_{\max} workload. In conclusion, in contrast to resting conditions, acetate recovery reaches a plateau value during exercise. Though this plateau value is repeatedly used to correct for label recovery in various exercise studies, our data clearly show that acetate label recovery during exercise not only depends on the exercise intensity but also on the applied infusion protocol. Therefore, theoretical

acetate recovery factors taken from previous literature are not generally applicable.

Keywords Exercise · Metabolism · Stable isotopes · Substrate utilization · Tracers

Introduction

In metabolic research, oxidation rates of substrates such as fatty acids, carbohydrate and amino acids are traditionally measured by quantifying the amount of labelled CO₂ produced following the administration of ¹³C or ¹⁴C-labelled substrates. The main assumption when using isotope tracer methodology is that the rate of appearance of labelled CO₂ in the expired breath accurately reflects the oxidation rate of the applied tracer. However, there is a need to correct for the proportion of labelled CO₂ that is produced via oxidation but not excreted in the expired breath. A substantial proportion of labelled CO₂ has been shown to become entrapped within the bicarbonate pool(s) of the body (Irving et al. 1983; Leijssen and Elia 1996). In addition, another substantial amount of label can be (temporarily) lost via isotopic exchange reactions in the tricarboxylic acid (TCA) cycle, mainly by way of conversion of α -ketoglutarate to glutamate and glutamine (Pouteau et al. 1998; Schrauwen et al. 1998). Sidossis et al. (1995a) were the first to propose that label retention can be quantitated by determining ¹³CO₂ (¹⁴CO₂) production during infusion of [¹³C]acetate (or [¹⁴C]acetate). Acetate, converted to acetyl-CoA, immediately enters the TCA cycle. Therefore, the fraction of acetate label retained in the body should equal the fraction of substrate label entrapped at any step between entering the TCA cycle and its appearance in expired CO₂. As such, an acetate correction factor is applied to correct simultaneously for the amount of label trapped as CO₂ in the bicarbonate pool(s), as well as for label fixated in other metabolites, via those isotopic exchange reactions (Sidossis et al.

L. J. C. van Loon (✉) · R. Koopman
P. Schrauwen · J. Stegen · A. J. M. Wagenmakers
Nutrition Research Institute Maastricht (NUTRIM),
Department of Human Biology,
Maastricht University, PO Box 616, 6200 MD
Maastricht, The Netherlands
E-mail: L.vanLoon@HB.Unimaas.nl
Tel.: +31-43-3881635
Fax: +31-43-3670976

1995a; van Hall 1999). Omitting to apply an acetate recovery factor leads to a substantial underestimation of plasma-derived fatty acid or glucose oxidation rates, especially during resting conditions.

We have validated the $[1,2-^{13}\text{C}]$ acetate recovery factor for correction of $[\text{U}-^{13}\text{C}]$ palmitate oxidation measured at rest (Schrauwen et al. 1998). As the position of the $[^{13}\text{C}]$ (or $[^{14}\text{C}]$) label in the applied tracer influences the amount of label retention (Baurle et al. 1998; Metges et al. 1994; Trimmer et al. 2001; Wolfe and Jahoor 1990), an $[1-^{13}\text{C}]$ acetate tracer should be used to correct oxidation rates of a $[1-^{13}\text{C}]$ glucose or fatty acid tracer, while $[1,2-^{13}\text{C}]$ acetate should be used in the case of a $[1,2-^{13}\text{C}]$ or $[\text{U}-^{13}\text{C}]$ tracer. As such, acetyl-CoA entering the TCA cycle will have ^{13}C atoms at the same position in both the acetate as well as substrate tracer infusion trial. At rest, we observed an intra-subject coefficient of variance (CV) in acetate label recovery of 4.0 (1.5)%, implying good reproducibility. However, inter-subject CV was 8.3 (0.6)%, suggesting that an acetate correction factor should be applied in each individual in parallel to the substrate infusion trial (Schrauwen et al. 1998, 2000).

At rest, whole-body acetate recovery increases with the duration of infusion (Mittendorfer et al. 1998; van Hall 1999). Mittendorfer et al. (1998) observed a continuous increase in acetate recovery up to 11 h after the onset of infusion. This implies that, during resting conditions, fractional acetate label recovery depends on infusion time and should be determined within the same period used to calculate substrate tracer oxidation rates. In contrast, during exercise acetate recovery has been shown to plateau, with its plateau value determined by exercise intensity (van Hall 1999). Subsequently, previously published acetate recovery factors are frequently used to correct substrate tracer oxidation rates in exercise studies. However, we hypothesize that pre-exercise infusion time also plays a major role in determining the absolute value of the obtained plateau in acetate recovery during exercise. We investigated the impact of both exercise intensity and infusion protocol on acetate label recovery during exercise. We, therefore, designed a number of different protocols which were applied in part to the same subjects to obtain additional data on intra-individual variance in acetate label recovery during exercise. The obtained data are used to provide sound advice on the proper use of the acetate recovery factor to accurately correct tracer oxidation rates for label retention in ongoing metabolic and clinical research.

Methods

Subjects

Eight trained cyclists [age: 22.1 (0.7) years; height: 1.86 (0.03) m; body mass: 74.5 (2.2) kg; W_{max} : 413 (9) W; $\dot{V}\text{O}_{2\text{max}}$: 5.5 (0.2) $\text{l}\cdot\text{min}^{-1}$] were selected to perform in protocol 1. Four of the subjects [age: 22.5 (0.5) years; height: 1.83 (0.02) m; body mass: 74.8 (2.7) kg; W_{max} : 427 (10) W; $\dot{V}\text{O}_{2\text{max}}$: 5.4 (0.2) $\text{l}\cdot\text{min}^{-1}$] volunteered to perform three additional trials in protocol 2 and another

additional trial in protocol 3. Subjects were informed about the nature and risks of the experimental procedures before their written informed consent was obtained. This study was approved by the local Medical Ethical Committee.

Pre-testing

Maximum workload (W_{max}) was measured on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands) during an incremental exhaustive exercise test 1 week before the first experimental trial (Kuipers et al. 1985). Findings were used to determine the 40, 55 and 75% W_{max} workload applied in protocols 1–3. Maximum oxygen uptake ($\dot{V}\text{O}_{2\text{max}}$) was measured to express the relative exercise intensity as % $\dot{V}\text{O}_{2\text{max}}$.

Diet and activity prior to testing

All subjects consumed a high carbohydrate meal [85 kJ/kg body mass⁻¹; consisting of 69 energy % (En%) carbohydrate, 15 En% fat and 16 En% protein] the evening before each trial. All subjects were instructed not to consume any products with a high natural abundance of ^{13}C (carbohydrate derived from C4 plants: corn, sugar cane) 1 week before and during the entire experimental period to minimize any shifts in background enrichment. They were also instructed to refrain from any exercise training 2–3 days prior to each trial.

Experimental trials

Protocol 1 was performed to study the effects of incremental exercise on acetate recovery. Therefore, subjects performed an incremental exercise protocol including three different workloads. In protocol 2, we determined the acetate recovery factor during exercise at each of those workloads separately, in the absence of any pre-exercise infusion period. Finally, in protocol 3 we further investigated the impact of the applied infusion protocol by determining the acetate recovery factor during the 40% W_{max} workload following an extended 4-h pre-exercise infusion period.

Protocol 1

The subjects arrived at the laboratory at 8.00 a.m. after an overnight fast. A Teflon catheter (Baxter BV, Utrecht, The Netherlands) was inserted in an antecubital vein for isotope infusion. After placement of the catheter subjects rested on a reclining chair and, approximately 20 min later, oxygen consumption and carbon dioxide production measurements were started and vacutainer tubes (Becton Dickinson, France) were filled directly from the mixing chamber in duplicate to determine background $^{13}\text{C}/^{12}\text{C}$ ratio in expired CO_2 . Immediately thereafter, subjects were administered a single intravenous dose of 0.064 $\text{mg}\cdot\text{kg}^{-1}$ of $\text{NaH}^{13}\text{CO}_3$ to prime the bicarbonate pool. At $t=0$ a constant intravenous infusion of $[1,2-^{13}\text{C}]$ acetate ($0.0736 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) was started and continued for 150 min. After 60 min acetate infusion rates were doubled ($0.1472 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) and subjects started to exercise on a cycle ergometer at an intensity of 40% W_{max} for a 30-min period. At $t=90$ exercise intensity was increased up to 55% W_{max} for another 30-min period. This was followed by a final 30-min period at 75% W_{max} . At rest $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured continuously during the first 60 min (Oxycon- β , Mijndhardt, Bunnik, The Netherlands). During exercise $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured every 5 min before breath sampling. Breath samples were collected at $t=0, 50, 55, 60$ min (rest); 80, 85, 90 min (40% W_{max}); 110, 115, 120 min (55% W_{max}) and finally 140, 145 and 150 min (75% W_{max}).

Protocol 2

Four of the cyclists participating in protocol 2 reported after an overnight fast at the laboratory on three additional occasions. Subjects were administered a single intravenous dose of $0.064 \text{ mg} \cdot \text{kg}^{-1} \text{ NaH}^{13}\text{CO}_3$. Immediately thereafter, exercise and the infusion of $[1,2-^{13}\text{C}]$ acetate (at the same infusion rate as applied in protocol 1: $0.1472 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) were started ($t=0$ min) and continued for up to 60 min. Exercise was performed on a cycle ergometer at an intensity of 40, 55 or 75% \dot{W}_{max} . $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured every 5 min before breath sampling. Breath samples were collected at $t=0, 10, 20, 30, 40, 50$ and 60 min. All trials were randomized and separated by at least 7 days.

Protocol 3

Cyclists that participated in protocol 2 reported after an overnight fast at the laboratory for an additional trial. After catheter insertion and background measurements (see protocol 1) subjects were administered a single intravenous dose of $0.064 \text{ mg} \cdot \text{kg}^{-1} \text{ NaH}^{13}\text{CO}_3$ to prime the bicarbonate pool. At $t=0$ a constant intravenous infusion of $[1,2-^{13}\text{C}]$ acetate (at the same resting infusion rate as applied in protocol 1: $0.0736 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) was started and continued for 240 min. After 240 min acetate infusion rates were doubled (at the same exercise infusion rates as applied in protocols 1–2: $0.1472 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) and subjects started to exercise on a cycle ergometer at an intensity of 40% \dot{W}_{max} for a 60-min period. Thereafter, acetate infusion was continued for an additional 60 min into the post-exercise period. $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured every 5 min before breath sampling. Breath samples were collected at $t=0, 60, 120, 180, 210, 220, 230, 235, 240, 245, 250, 260, 270, 285, 300, 305, 310, 315, 330$ and 360 min.

Analysis

The acetate tracer (sodium salt of $[1,2-^{13}\text{C}]$ acetate, 98.9% enriched; Cambridge Isotope Laboratories, Andover, Mass., USA) was dissolved in 0.9% saline. The chemical and isotopic purity (98.9%) of the acetate tracer was checked by ^1H and ^{13}C NMR and GC/MS. In each infusate the exact acetate concentration was measured using an enzymatic method (Boehringer, Mannheim, Germany). Breath samples were analysed for $^{13}\text{C}/^{12}\text{C}$ ratio by gas chromatography isotope ratio mass spectrometry (GCIRMS) (Finnigan MAT 252, Bremen, Germany).

Calculations

The ^{13}C enrichment of breath CO_2 is presented as tracer/tracee ratio (TTR)

$$\text{TTR} = (^{13}\text{C}/^{12}\text{C})_{\text{sa}} - (^{13}\text{C}/^{12}\text{C})_{\text{bk}} \quad (1)$$

in which sa indicates sample and bk indicates background value. Fractional recovery of label in breath CO_2 , derived from the infusion of labelled acetate, was calculated as follows:

$$\text{Fractional recovery of label (\%)} = [(\text{TTR } \text{CO}_2 \cdot \dot{V}\text{CO}_2)/2F] \cdot 100\% \quad (2)$$

where TTR CO_2 is tracer/tracee ratio (TTR) in breath CO_2 , $\dot{V}\text{CO}_2$ is carbon dioxide production ($\text{mmol} \cdot \text{min}^{-1}$) and F is the infusion rate of $[1,2-^{13}\text{C}]$ acetate ($\text{mmol} \cdot \text{min}^{-1}$).

Statistics

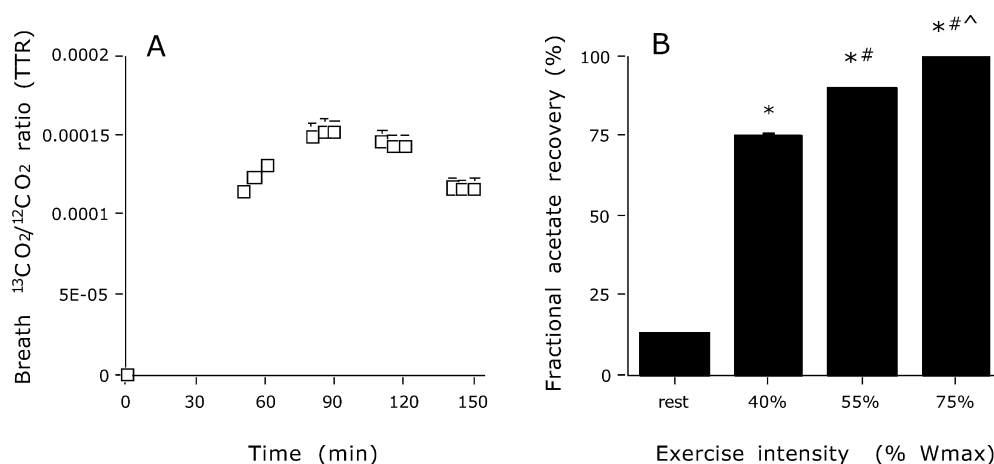
All data are expressed as mean (SE). Statistical analysis of the data collected was done using a two-factor analysis of variance (ANOVA). Differences in acetate label recovery during exercise of various intensities was checked for statistical significance using a Scheffé post hoc test. In addition, simple regression analysis was performed to calculate correlation between the oxygen uptake/pre-exercise infusion duration and fractional acetate label recovery and between acetate recovery factors obtained in protocols 1–3. Statistical significance was set at $P < 0.05$.

Results

Protocol 1

Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios are shown in Fig. 1A. Ratios increased over time under resting conditions (0–60 min). During exercise, ratios remained constant over the last 10–15 min of each exercise stage. Label recovery averaged 13.7 (0.4)% at the end of the resting period. Acetate recovery was significantly higher during exercise and averaged 75.1 (1.6), 91.2 (0.7) and 101.1 (1.9)% at the end of the 40, 55 and 75% \dot{W}_{max} workload, respectively. Fractional acetate recovery was significantly different between the various exercise intensities (Fig. 1B). $\dot{V}\text{O}_2$ averaged 2.41 (0.07), 3.11 (0.08) and 3.91 (0.11) $\text{l} \cdot \text{min}^{-1}$ [44.1 (1), 56.8 (0.8) and 71.6 (1.6)% $\dot{V}\text{O}_{2\text{max}}$] during the last 15 min of the 40, 55 and 75% \dot{W}_{max} exercise stage, respectively. Simple regression showed a significant correlation between $\dot{V}\text{O}_2$ and acetate recovery ($R = 0.80$; $P < 0.0001$). Inter-subject CV for acetate label recovery

Fig. 1A, B Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios over time (A) and calculated fractional acetate recovery (B) at rest and during incremental exercise at an exercise intensity of 40, 55 and 75% of the maximal workload (\dot{W}_{max}), respectively, during continuous intravenous infusion with $[1,2-^{13}\text{C}]$ acetate [means (SEM)]. *Significant difference compared to resting conditions; #significant difference compared to 40% \dot{W}_{max} ; ^significant difference compared to 55% \dot{W}_{max} ($P < 0.05$)



at rest and during exercise were 12.2% and 7.6%, respectively.

Protocol 2

Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios over time during exercise at an intensity of 40, 55 and 75% W_{max} are illustrated in Fig. 2A. Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios increased over time and in the absence of a pre-exercise infusion period did not reach a plateau value within 30–60 min. In the 75% W_{max} trial subjects exercised until exhaustion which turned out to be less than 40 min in three subjects. Subsequently, ratios are only reported up to 30 min at the 75% W_{max} workload. Over the last 30 min fractional acetate recovery averaged 66.9 (3.2) and 71.2 (1.3)% in the 40 and 55% W_{max} trial, respectively (Fig. 2B). In the 75% W_{max} trial fractional acetate recovery increased up to an average of 74.9 (6.0)% after 30 min. Differences in fractional acetate recovery rates between the different exercise intensities within this protocol did not reach statistical significance, but fractional acetate recovery rates at each workload (in the absence of pre-exercise label infusion) were substantially lower than recovery rates observed following 1 h of pre-exercise infusion

(protocol 1; $P < 0.05$). Inter-subject CV for acetate label recovery during exercise averaged 9.8%.

Protocol 3

Breath TTR ratios over time at rest and during exercise at an intensity of 40% W_{max} are illustrated in Fig. 3A. Ratios increased gradually at rest over the entire 4-h period. At the onset of exercise, ratios decreased and gradually reached plateau values after 60 min of exercise. After exercise, ratios started to increase over time during post-exercise recovery. Fractional acetate label recovery (Fig. 3B) increased up to 36.9 (2.0)% at rest, after 4 h of infusion. After onset of exercise, label recovery increased up to 142.2 (2.2)% and gradually reached a plateau at 97.6 (6.0)% during the last 10 min of exercise. In the post-exercise stage, acetate label recovery dropped to 10.5 (1.1)% within 5 min, after which label recovery gradually increased. Inter-subject CV for acetate label recovery at rest and during exercise was 11.7% and 10.5%, respectively.

Protocols 1–3

Fig. 2A, B Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios over time (A) and calculated fractional acetate recovery (B) during exercise at different intensities (40, 55 and 75% W_{max}) following continuous intravenous $[1,2-^{13}\text{C}]$ acetate infusion, started immediately at the onset of exercise without any pre-exercise infusion period. *Significantly lower value compared to fractional acetate recovery rates observed with pre-exercise infusion period (protocol 1) ($P < 0.05$)

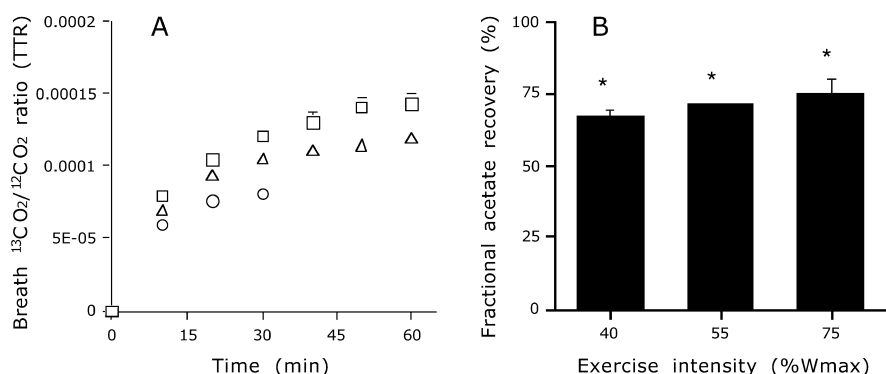
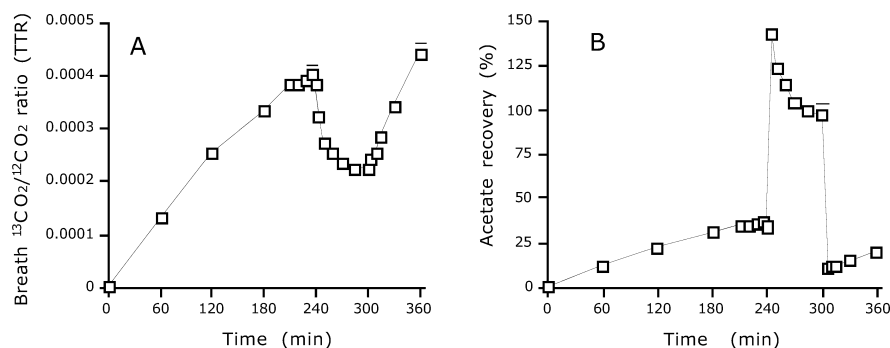


Fig. 3A, B Breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios over time (A) and calculated fractional acetate recovery (B) at rest, during moderate intensity exercise (40% W_{max}) and in the post-exercise resting phase following continuous intravenous $[1,2-^{13}\text{C}]$ acetate infusion, started 4 h before the onset of exercise



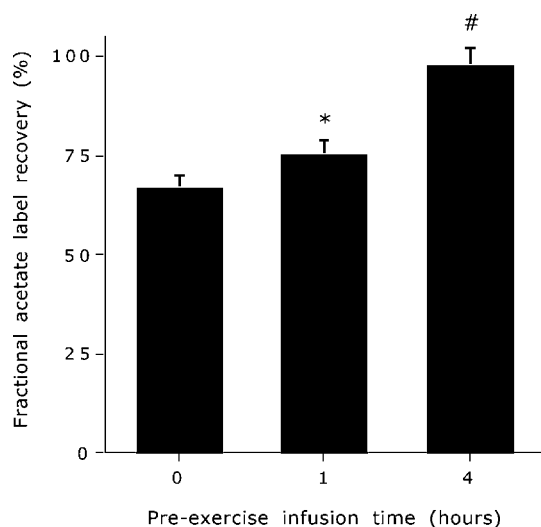


Fig. 4 Fractional acetate recovery rates during moderate intensity exercise (40% \dot{W}_{\max}) following continuous intravenous [1,2- ^{13}C]acetate infusion, started immediately at the onset of exercise, following 1 h and following 4 h of pre-exercise infusion. *Significantly higher compared to protocol 1 (0 h); #significantly higher compared to protocol 2 (1 h) ($P < 0.05$)

infusion averaged 66.9 (3.2), 75.1 (1.6) and 97.6 (6)%, respectively ($P < 0.05$). Regression analysis showed a significant positive correlation between the pre-exercise acetate label infusion period and the fractional acetate label recovery during the subsequent exercise stage ($P < 0.001$). When the individual acetate recovery factors obtained at the 40% \dot{W}_{\max} workload in protocol 1 were compared in a scatter plot to the values obtained in protocols 2 and 3, strong correlations were found; $R = 0.98$ and 0.96 ($P < 0.05$), respectively. Inter-individual CV in acetate recovery during exercise in protocols 1–3, averaged 9.0 (1.6)%.

Discussion

When carbon-labelled glucose or fatty acid tracers are used to determine substrate oxidation rates, corrections should be made for labelled CO_2 that is produced but not excreted as well as for label fixated in other metabolites (Schrauwen et al. 1998; Sidossis et al. 1995a; van Hall 1999). An acetate correction factor has been designed to provide a practical means to correct for such label retention (Sidossis et al. 1995a; Tounian et al. 1996). Omitting to apply an acetate correction factor leads to substantial underestimation of plasma-derived glucose or fatty acid oxidation rates (Mittendorfer et al. 1998; Schrauwen et al. 1998, 2000; Sidossis et al. 1995a). When implementing the use of [^{14}C]acetate, both acetate and the ^{13}C substrate tracer can be applied within one single trial, but in many places medical ethical considerations limit the use of radioactive tracers. As such, when using stable isotope methodology to determine substrate tracer oxidations rates, a separate acetate

recovery trial needs to be performed in parallel with the tracer infusion trial to correct for carbon label retention. We studied the effects of both exercise and infusion protocol on ^{13}C label recovery following continuous [1,2- ^{13}C]acetate infusion.

First, we measured acetate recovery rates during incremental exercise to determine the effects of exercise intensity on label recovery within a single trial. At rest, acetate recovery increased continuously. In contrast, during exercise acetate recovery reached a plateau value after 15 min during each exercise stage (Fig. 1A). The plateau values in fractional acetate recovery rates were significantly different between the three exercise stages (Fig. 1B). During exercise at the 75% \dot{W}_{\max} workload, acetate recovery actually reached a plateau at $\sim 100\%$, implying no need for a correction factor for label retention. In line with findings on label recovery following [1- ^{14}C]acetate infusion (Sidossis et al. 1995a), we observed a strong correlation between [1,2- ^{13}C]acetate label recovery and $\dot{V}\text{O}_2$ ($R = 0.8$; $P < 0.0001$). Sidossis et al. (1995a) reported label recovery to range between 66 and 94%, as exercise intensity varied between 23 and 81% peak $\dot{V}\text{O}_2$ in 11 random subjects.

The increase in acetate recovery with increasing $\dot{V}\text{O}_2$ is explained by the fact that there is less chance for label to be fixated via TCA cycle exchange reactions at a higher workload. This is a consequence of the fact that TCA-cycle flux increases in proportion to exercise intensity, while exchange reactions (e.g. glutamine production in muscle) maintain a constant pace. In the study by Sidossis et al. (1995a), 100% label recovery was not obtained, though exercise intensities as high as 81% of peak $\dot{V}\text{O}_2$ were applied. The latter can be explained by the inclusion of trained cyclists in the present study, who are able to cycle at much higher absolute workloads and concomitant $\dot{V}\text{O}_2$ rates [3.9 (0.1) $\text{l}\cdot\text{min}^{-1}$], enabling us to demonstrate that full 100% label recovery can be achieved.

Our findings, in accordance with others (van Hall 1999), show that, in contrast to resting conditions, acetate label recovery tends to plateau during exercise with its plateau value being determined by the applied exercise intensity (Schrauwen et al. 2000; Sidossis et al. 1995a). Subsequently, published acetate recovery factors are currently used to correct fatty acid tracer oxidation rates in exercise studies. Whether this is a valid approach has never been thoroughly investigated. Schrauwen et al. (2000) recently reported an inter-subject CV in acetate label recovery of 12% at rest and 16% during a subsequent exercise trial. In the present study, in a more homogenous group of cyclists, inter-subject CV was 12% and 8%, respectively (protocol 1). In comparisons between studies, differences in reported acetate recovery rates during exercise are often much larger than the reported inter-subject CV of 8–16%, which cannot entirely be explained by differences in exercise intensity, body composition of the subjects and/or specific tracers used. We hypothesized that acetate label recovery during exercise not only depends on exercise intensity but also

on the applied infusion protocol. As we have shown that a substantial proportion of label is lost by way of conversion of α -ketoglutarate to glutamate and glutamine (Schrauwen et al. 1998; Sidossis et al. 1995a, 1995b), it could be hypothesized that differences in infusion protocol could lead to the accumulation of label in the glutamate/glutamine pools. As such, preceding infusion time determines the extent to which the glutamine and glutamate pools will become labelled and subsequently oxidized. Previous research (Gibala et al. 1997; Sahlin et al. 1995; van Hall et al. 1995) has shown that the muscle glutamate pool is reduced during exercise in proportion to exercise intensity, as this pool is used for the generation of TCA cycle intermediates. This implies that more $^{13}\text{CO}_2$ will be generated during exercise when the muscle glutamate pool becomes more enriched following a longer pre-exercise infusion period. As such, with increasing (pre-exercise) infusion time, the extent of label recovery during exercise and the subsequent plateau value in fractional label recovery could be affected.

To test this hypothesis, we determined acetate label recovery during exercise at 40, 55 and 75% W_{max} workload in separate trials with the omission of any pre-exercise infusion time (protocol 2). In agreement with the hypothesis, lower acetate recovery rates were obtained using protocol 2 compared to protocol 1 at each separate workload (Figs. 1, 2). Clearly, the reduced recovery rates as well as the less pronounced differences in label recovery between the different exercise intensities are well in accordance with our hypothesis. In the absence of any pre-exercise acetate infusion, less label is incorporated into the glutamate pool. Subsequently, during exercise less $^{13}\text{CO}_2$ is generated following reduction of the glutamate pool. In addition, as the glutamate pool is reduced in proportion to exercise intensity, absolute differences in acetate label recovery during exercise of various intensities will depend on the extent of ^{13}C enrichment of the glutamate pool.

To further validate our hypothesis we extended the pre-exercise infusion period to 4 h followed by exercise at the 40% W_{max} workload (under identical infusion conditions as used in protocols 1–2). Following the onset of exercise, acetate recovery increased up to 142 (2)%, after which it declined, reaching a plateau value of ~100% (Fig. 3). These data provide further proof of the proposed relationship between pre-exercise infusion time and acetate label recovery during exercise. As the glutamine/glutamate pool becomes substantially enriched with ^{13}C label during the extensive 4-h resting period, more $^{13}\text{CO}_2$ is recovered with the onset of exercise when the glutamate pool declines. As such, acetate recovery temporarily increases above 100% as the excessive amount of entrapped label is released. Within 60 min a new equilibrium is attained showing full ~100% label recovery. The impact of the infusion protocol on fractional acetate label recovery during exercise is illustrated in Fig. 4, and reveals a strong positive correlation between pre-exercise infusion time and the obtained plateau value in label recovery ($P < 0.001$). Regression

analyses, comparing acetate label recovery rates observed in protocol 1 with those seen in protocol 2 and 3, approached 1.00 ($R = 0.98$ and 0.96 , respectively; $P < 0.05$). This indicates that there are individual factors which determine whether subjects have a high or low acetate label recovery in repeated exercise tests performed under different experimental conditions. The overall inter-subject CV on label recovery during exercise averaged 9.0 (1.6)% in the present study, confirming and extending our previous conclusions (Schrauwen et al. 1998, 2000) that there is a need for a separate acetate recovery trial within each individual subject even when (pre-)infusion conditions do not vary.

In summary, to accurately correct ^{13}C tracer oxidation rates for carbon label retention, an acetate recovery factor needs to be applied. Omitting to apply an acetate correction factor leads to a gross underestimation of tracer oxidation rates at rest and, in most cases, a substantial underestimation during exercise. In contrast to resting conditions, acetate label recovery reaches an apparent plateau value during exercise. Though values obtained from previously published studies are repeatedly used to correct for label retention in studies performed in other laboratories and/or under different conditions, our data clearly show that acetate label recovery during exercise depends on the applied exercise intensity, pre-exercise infusion time as well as on the individual subjects used in the studies. As such, theoretical acetate recovery factors should not be generally used. Our advice is that an acetate correction test should be determined in every subject, for the same period (at rest) and preceded by the same pre-exercise infusion protocol and workload (during exercise conditions) as applied in the substrate tracer infusion trial.

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